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Since this grant was awarded (7/1/96), we have constructed a set of deletion mutants and showed that deletion of last 30 amino acids in p53 severely disrupted its ability to bind to c-Abl and deletion of the tetramerization domain also greatly reduced the binding to c-Abl. Based on these results, we proposed a model in which c-Abl interacts with the regulatory domain (aa 363 to 393) on p53 to diminish its negative regulatory effect and thereby to enhance the DNA binding activity of p53. This interaction, however, requires the tetrameric conformation of the protein. To test this, we first investigated the ability of a mutant p53 (341K344E348E355K, tetramerization impair) to interact with c-Abl and showed that this mutant is defective in c-Abl interaction. Second, we proposed to obtain the purified c-Abl protein via a baculovirus expression system to assay its ability to enhance p53's DNA binding. Unfortunately, we have not been able to do so using c-Abl virus we have. We are currently in a process to construct a new virus which expresses His-tag c-Abl. We hope that we will be able to obtain a large amount of purified protein to enable us to perform EMSA experiments as we proposed.

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Introduction

The loss of cell growth regulation is a hallmark of cancer. To achieve our goal of designing therapies for cancer, we must understand how cancer proteins affect cell growth. The aim of this proposal is to address this question for the cancer related proteins, p53 and c-Abl. Our previous work demonstrated that c-Abl requires p53 for growth suppression. In studying the mechanism of this effect, we find that c-Abl can enhance the transcriptional activity of p53 *in vivo*. This enhancement requires a domain in c-Abl that mediates binding to p53 (Goga, Liu et al, 1995). Because we are unable to observe a direct phosphorylation of p53 by c-Abl, we hypothesize that c-Abl may function through the following possible mechanisms to activate p53-dependent transcription: 1) c-Abl enhances the DNA-binding activity of p53; 2) c-Abl brings other factors to the promoter; 3) c-Abl phosphorylates general transcription machinery, which in turn allows p53 to function. In keeping with this trend, we have proposed:

I. To define the domains on p53 required for c-Abl binding
2. To examine the effect of c-Abl on the DNA-binding activity of p53
3. To characterize the effect of c-Abl on p53-dependent transcription *in vitro*
4. To determine the effect of Gal4-Abl on transcription from a promoter containing Gal4 sites
5. To examine whether general transcription factors are phosphorylated by c-Abl

Background and Previous Work

p53 is an important tumor suppressor gene, mutated, or absent, in over 50% of all cancers studied (Hollstein *et al.*, 1991, Levine *et al.*, 1991). It functions as a sequence-specific DNA-binding transcription factor (Bargonetti *et al.*, 1991; Farmer *et al.*, 1992). In response to double-stranded DNA breaks (Nelson and Kastan, 1994) p53 is converted from a latent to an active form (Hupp *et al.*, 1992). This results in increased expression of p53-responsive proteins such as p21 (El-Diery *et al.*, 1993; Xiong *et al.*, 1993; for review, see Ko and Prives, 1996) which are required for growth arrest at the G1 to S phase transition (Kastan *et al.*, 1992; Lu and Lane, 1993). It also mediates apoptosis via the increased expression of proteins such as Bax (Miyashita and Reed, 1995). Inactivation of p53, therefore, results in the loss of a cell cycle checkpoint required for repair of damaged DNA and prevents apoptosis in response to severe DNA damage. In the absence of these responses oncogenic mutations can accumulate which may result in tumor progression.

Based on the G1 arrest phenotype of p53, we reasoned that p53 transcriptional activity must be affected by the cell cycle proteins which regulate G1. c-Abl has been reported to be a growth suppressor and overexpression of c-Abl leads to G1 growth arrest in fibroblasts (Sawyers et al, 1994). The c-Abl protein is a predominantly nuclear tyrosine kinase. The kinase activity of c-Abl is tightly regulated *in vivo*, possibly by binding to unidentified inhibitory proteins (Pendergast et al,

1991), and is required for c-Abl to suppress growth (Sawyers et al, 1994). Links between the c-Abl proto-oncogene and cell cycle suggest that c-Abl normally acts as a negative regulator of cell growth and that it may function through p53. The availability of mouse fibroblasts containing disruptions of the Rb or p53 genes allowed us to genetically test this possibility. Our results show that c-Abl requires p53 but not Rb to suppress growth. In addition, we also find that c-Abl binds to p53 *in vitro* and enhances the ability of p53 to activate transcription from a promoter containing a p53 DNA binding site in a transient transfection assay. Deletion of the p53 binding domain in c-Abl (ΔProl, a deletion of proline rich domain, aa 793-1044) impairs the ability of c-Abl to stimulate p53 transcriptional activity and to suppress growth (Goga, Liu et al, 1995). These results suggest that the regulation of p53 transcription is very important in negative growth control by c-Abl. Therefore, a detailed understanding of how c-Abl stimulates p53-dependent transcription may allow the rational design of therapies which can reactivate the Abl-p53 pathway in tumor cells, resulting in cell cycle arrest and apoptosis of tumor cells.

Towards this end, during the first year of the grant (7/96 to 6/97), we constructed a set of deletions to disrupt the domains of p53 responsible for nuclear localization (aa 316 to 322), tetramerization (aa 325 to 356) and the regulation of the DNA binding activity of p53 (aa 363 to 393). Furthermore, the ability of these mutants to interact with c-Abl was also tested using a GST pull-down assay. Our results show that deletion of last 30 amino acids in p53 severely disrupted its ability to bind to c-Abl and deletion of the tetramerization domain also greatly reduced the binding to c-Abl. Based on these results, we propose a model in which c-Abl interacts with the regulatory domain (aa 363 to 393) in p53 to diminish its negative regulatory effect and to enhance the DNA binding activity of p53. This interaction, however, requires the tetrameric conformation of the protein. To test this requirement, ability of a mutant p53 (341K344E348E355K, tetramerization impair) to interact with c-Abl was investigated. Our results show this mutant is defective in c-Abl interaction. These data led us to focus our studies on c-Abl's ability to enhance the DNA binding activity of p53.

Experimental Methods and Procedures

Baculovirus Expression and purification of c-Abl and GST-Abl proteins

c-Abl was prepared from SF21 insect cells infected with recombinant baculovirus (a gift from Dr. O. Witte, UCLA) as described by Pendergast *et al.* (1). Briefly, Sf21 cells (1×10^6) were infected with the recombinant baculovirus and cultured in suspension (200 ml). At 3 day post-infection the cells were resuspend in lysis buffer (25 mM KPO_4, pH 7.0, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 5 mM PMSF and 50 μg/ml leupeptin) and lysed by 20 strokes using a Dounce homogenizer. The cell lysates were centrifuged for 10 min at 3000 g and the supernatants were resedimented at 100,000 g for 90 min. The high speed supernatant was applied to a 10 ml BioRex-

70 columns equilibrated with buffer E (25 mM KPO_4, pH 7.0, 2 mM EDTA, 5 mM PMSF and 50 µg/ml leupeptin) containing 150 mM NaCl. The column was washed with the same buffer and c-Abl eluted with 500 mM NaCl buffer E. The proteins were dialyzed against buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing150 mM KCl . The dialyzed proteins were then applied onto a FPLC Mono-Q column and eluted with a 15 ml of gradient (100 mM - 1M KCl) in D buffer. GST-Abl cell lysate was prepared from SF21 insect cells infected with recombinant baculovirus (gifts from Dr. O. Witte, UCLA) as above. The lysate was then incubated with 0.3 ml of GST-Sepharose beads and the GST-Abl proteins were eluted with 10 mM reduced Glutathion.

p53 Purification

p53 was immunopurified from nuclear extracts prepared according to the method of Dignam *et al.* (10). One milliliter of nuclear extract was incubated for 6 hours at 4 °C with gentle rotation with 100 µl of packed protein A-Sepharose beads, to which Pab 421, a monoclonal antibody specific for p53, was covalently linked. Beads were washed twice with 0.5 M KCl D buffer and once with 0.1 M KCl D buffer. p53 was eluted from the washed beads with 100 µl 421 epitope oligopeptide (KKGQSTSRHKK) at 1 mg/ml concentration in 0.1 M KCl D buffer. Proteins were analyzed by electrophoresis in a 10% SDS-polyacrylamide gel which was subjected to silver staining to visualize bands.

Electrophoresis Mobility Shift Analysis (EMSA)

The oligonucleotide probe containing the ribosomal gene cluster (RGC) p53-binding site is as follows: 5'- AGCTTGCCTCGAGCTTGCCTGGACTTGCCTGGTCGACGC - 3'. Binding reactions contained 60 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 10 µg BSA, 0.5 g poly [d(GC)], 200 pg ^{32}P-labeled probe, proteins as indicated, and water in a total volume of 12.5 µl. Reactions were incubated for 30 minutes at 30 °C and then analyzed on a 3% polyacrylamide gel containing 0.5 x TBE (0.045 mM Tris-borate, 0.045 mM sodium borate, 0.001 mM EDTA [pH 8.0]). Electrophoresis was carried out for 2 hours at room temperature in 0.5 x TBE. The gel was dried and DNA-protein complexes were visualized with a PhosphorImager using Adobe Photoshop software.

Results and Discussion

I. Partially purified c-Abl stimulates p53 DNA-binding activity:

We expressed c-Abl with baculovirus system and purified it using BioRex70 and Mono Q column. The purification is sown in Figure 1 and the partially purified protein after Mono Q column is shown in Figure 2.

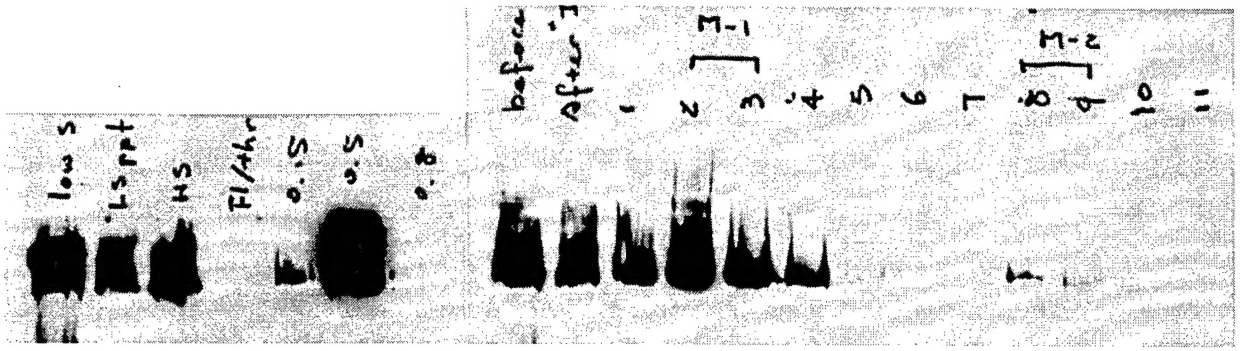

Figure 1. c-Abl purification. A). Western blot analysis showing that c-Abl is present in E0.5 (lane 6) and Mono Q (lanes 11 – 13; M-1) fractions.

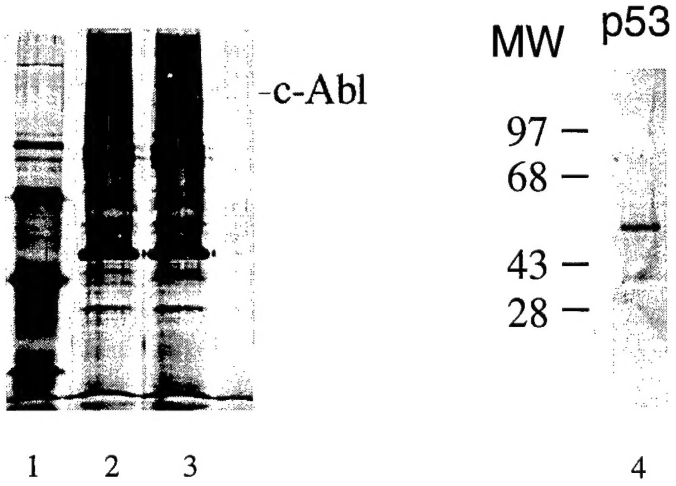

Figure 2. Silver staining gel showing partially purified c-Abl preparation. Lane 1: mol wt. marker; lanes 2 and 3: two-column purified c-Abl; Lane 4: the purifed p53 protein.

We tested the partially purified c-Abl for its ability to stimulate p53 DNA binding activity using EMSA with a probe containing the p53 *cis* element identified in the ribosomal gene cluster (RGC). We have shown previously that vaccinia virus expressed human p53 purified from HeLa cells (Figure 2, lane 4) can specially bind this element and produced a retarded p53/DNA complex (Figure 3, lanes 1 and 2) which was supershifted by the addition of anti-p53 antibody N-19 (data not shown). When the partially purified c-Abl was added to this reaction, an enhanced p53-DNA complex with similar mobility to those formed by the vhp53 (cf. lanes 1, 2, 6 and 7) was observed. As a control, an identified fraction purified from mock infect cells did not enhance p53's DNA binding activity under the same condition (cf. lanes 4 and 5). This result suggested that the DNA binding ability of p53 was enhanced by partially purified c-Abl fraction.

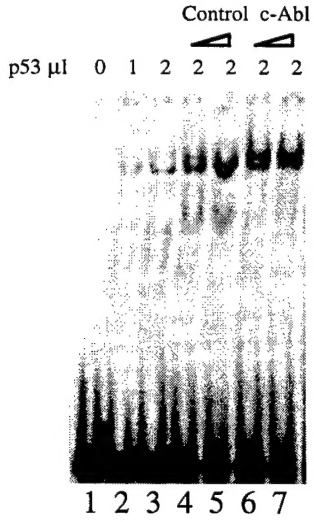

Figure 3. c-Abl enhances the DNA binding activity of c-Abl. EMSA was performed by electrophoresis in a 4% PAGE gel in 0.5X TBE. The volume of purified p53 added is indicated in microliters. Increased amounts of baculovirus expressed and partially purified c-Abl were added to lanes 6 and 7 and the same amounts of control extract were added to lanes 4 and 5.

II. We are currently working to obtain highly purified c-Abl protein:

In order to test our model that c-Abl interacts with the negative regulatory domain to enhance the DNA binding activity of p53, we proposed to obtain the purified c-Abl protein via a baculovirus expression system. Unfortunately, we have not been able to obtain purified protein using the virus we have. We are currently in a process to construct a new virus which expresses His-tag c-Abl to facilitate the purification. We hope that we will be able to obtain a large amount of purified protein to enable us to test our model.

Recommendation in Relation to the Statement of Work

As discussed above, our preliminary experiments have demonstrated that c-Abl interacts with the negative regulatory domain of p53. Based on this result, Task 2, 5 and 6 will not be performed. We will focus our research on obtaining a large amount of c-Abl protein, so that we can perform detailed EMSA experiments. For this purpose, a postdoctoral fellow, Dr. Nie who has expertise in protein purification and baculovirus expression, has joined us from UCLA. In addition, we just will also plan to perform experiments described in Aim 4. Specifically:

Task 1: Done

Task 2: Information is available in the literature

Task 3 and 4: Work in progress

Task 5 and 6: will not be performed based on our results

Task 7 and 8: Work in progress

Task 9:

Conclusions

We have demonstrated an interaction between c-Abl and p53 c-terminal regulatory region. Based on this result, we propose a model in which c-Abl interacts with the negative regulatory domain to enhance the DNA binding activity of p53. Unfortunately, we have not been able to obtain a large amount of purified c-Abl protein to test our model. Using partially purified c-Abl fraction, an enhanced DNA binding activity of p53 was observed. We are currently in a process to construct a new virus which expresses His-tag c-Abl to facilitate the purification. We hope that we will be able to obtain enough purified protein using this virus.

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